Document 1

Differential Expression of HLA-E, HLA-F, and HLA-G Transcripts in Human Tissue

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ABSTRACT: *The data presented here demonstrate that the HLA-G class I gene is unique among the members of the human class I gene family in that its expression is restricted to extraembryonic tissues during gestation. Furthermore, the pattern of HLA-G-expression in these tissues changes as gestation proceeds. During first trimester HLA-G is expressed within the placenta and not within the extravillous membrane. At term, the pattern of the HLA-G expression is reversed, extravillous membrane expresses HLA-G while placenta does not. Another non-HLA-A, -B, -C class I gene, HLA-E, is also expressed by extraembryonic tissues. Unlike HLA-G, HLA-E is expressed by both placenta and extravillous membrane at first trimester and at term. These results raise the intriguing possibility that the HLA-G-encoded molecule has a role in embryonic development and/or the fetal—maternal immune response.*

ABBREVIATIONS

bp	base pair	β_2m	β_2-microglobulin
B-LCL	B-lymphoblastoid cell line	nt	nucleotides
MHC	major histocompatibility complex	T-LCL	T-lymphoblastoid cell line

INTRODUCTION

The human major histocompatibility complex (MHC), the HLA complex, is a genetic region located in the short arm of chromosome 6. To date, the most extensively characterized members of the HLA class I gene family are the genes encoding the major transplantation antigens, HLA-A, -B, and -C. These genes encode highly polymorphic 44-KD heavy chains that are noncovalently associated with an invariant 12-KD subunit, β_2-microglobulin (β_2m), at the cell surface [1]. The major transplantation antigens function to present antigens to cytolytic T lymphocytes. Cloning and characterization of human class I sequences has resulted in the identification of three HLA non-A, B, C class I genes, designated HLA-E [2,3], HLA-F [4], and HLA-G [5]. Genomic clones for each have been sequenced and found to encode intact HLA class I heavy chains distinct from either HLA-A, -B, or -C. DNA-mediated gene transfer of HLA-E, -F, and -G into a human mutant B-lymphoblastoid cell line (B-LCL) that no longer expresses its endogenous HLA-A, -B, and -C antigens resulted in the expression of HLA class I heavy chains that could associate with β_2m [6]. However, only the protein encoded by

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the HLA-G gene formed a complex with β_2m that was detectable on the cell surface. The HLA-E and -F complexes with β_2m, yet failed to be expressed on the cell membrane of the B-LCL.

Analysis of the tissue and cellular pattern of gene expression is a means by which insight into biologic function of the gene product might be gained. Northern blot analysis of HLA-E and RNase protection studies of HLA-F indicated that both genes were expressed [3,4]. In this study, gene-specific RNase protection assays are used to extend the earlier study on HLA-E and to include HLA-G. The results demonstrate that among these three non-A, B, C class I genes the expression of the HLA-G gene is uniquely restricted to the extraembryonic tissues. Moreover, the pattern of HLA-G expression in these tissues changes with the progression of gestation.

MATERIALS AND METHODS

Cells and culture. The human leukemic T-cell lines, Molt-4 [7], CEM [8], HSB-2 [9], and Hut-78 [10] were obtained from the American Type Culture Collection. These cells and Ebstein-Barr-virus-transformed lymphoblastoid cell lines 721 [11], and FJO [12] were grown in RPMI-1640, 10% fetal bovine serum (GIBCO), and 5% CO_2 at 37°C. Cell densities were maintained between 0.5 and 1.0 × 10^6 cells/ml. To prepare T-lymphocyte blasts, mononuclear cells were isolated from heparinized peripheral blood of normal healthy donors by centrifugation on Ficoll-Hypaque and cultured in 1% phytohemagglutinin (PHA) for 60 hr before isolation of cellular RNA. Resting T lymphocytes were prepared by passage of peripheral blood mononuclear cells depleted of monocytes through a nylon wool column.

Tissues. Human skin and liver were from the National Disease Research Interchange, Philadelphia, and from the Division of Surgical Pathology of the Department of Laboratory Medicine and Pathology, University of Minnesota. Term placental tissue was obtained from normal deliveries as discarded tissue through the Department of Obstetrics and Gynecology of the University of Minnesota. Extraembryonic tissues from first trimester and second trimester were obtained from elective pregnancy terminations. All tissues were processed immediately upon arrival in the laboratory. Tissues were first rinsed in a large volume of cold phosphate-buffered saline. Small fragments were then dissected from placentas and attached extravillous membranes of the 21st week of gestation and the term material. In case of the 10th week of gestation, villi adjacent to the decidua basalis are enlarged in size and increased in number while villi in the portion of the chorion facing the decidua capsularis are very few in number and short in length. Fragments dissected from the villi-rich area of the 10th-week extraembryonic membrane were labeled as placenta, and fragments dissected from the 10th-week villi-poor region were labeled as extravillous membrane. All villi from the 10th-week extravillous membrane were trimmed away.

RNA isolation. Total cellular RNA was isolated from cells and tissues by the guanidinium thiocyanate precipitation method of Chirgwin et al. [13] and pelleted through a cesium chloride cushion. RNA samples were assessed for degradation on a 1% agarose mini-gel prior to use in RNase protection assays.

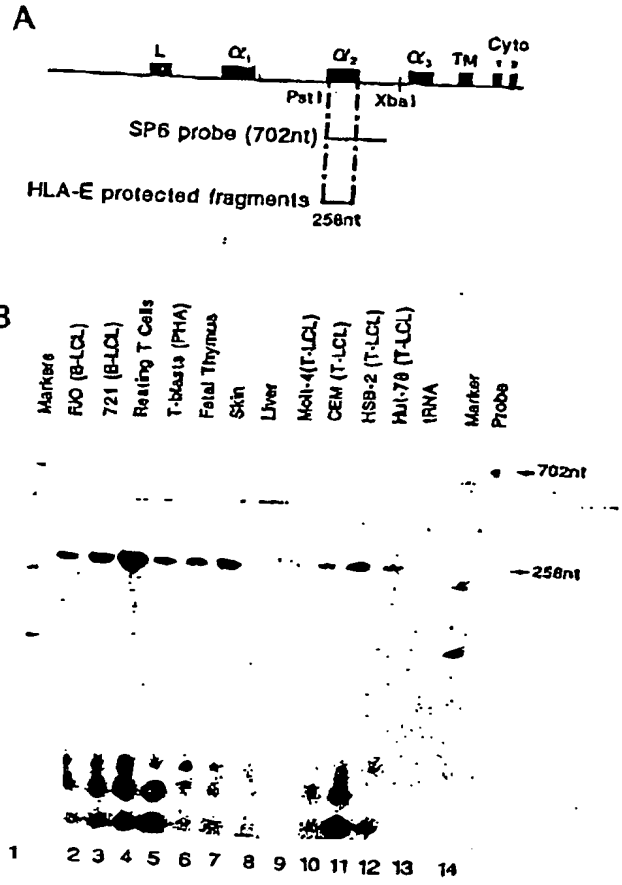

FIGURE 1 HLA-E RNA expression in human cells and tissues. (A) Diagram of the Sp6 antisense RNA probe used for detecting HLA-E transcript by RNase protection. Full-length antisense HLA-E RNA probe was 702 nt in length. Protection by HLA-E mRNA yields a fragment 258 nt in length. (B) ^{32}P-labeled antisense probe was hybridized to 12 μg of RNA, digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis (lanes 2–12). Lane 13 depicts probe hybridized to tRNA alone. Arrows indicate positions of the full-length (702-nt) antisense probe and the 258-nt HLA-E protected fragment (258 nt).

RNase protection assays. Synthesis of ^{32}P-radiolabeled antisense RNA probes and RNA protection analyses were carried out according to Melton et al. [14]. Linearized DNA was used as a template for the synthesis of [^{32}P] CTP uniformly labeled antisense RNA probes by SP6 RNA polymerase. After elongation, the DNA was removed with DNaseI and the labeled RNA purified by ethanol precipitation twice. Twelve micrograms of total RNA and the labeled probe (4×10^5 CPM) were mixed in 30 μl of buffer containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4), and 1.0 mM EDTA, heated at 85°C for 10 min and incubated at 50°C overnight. Digestion was initiated by the addition of 300 μl of buffer containing 0.01 M Tris (pH 7.5), 0.3 M NaCl, 5.0 mM EDTA, 40 μg/ml RNase A, and 2 μg/ml RNase T1. Samples were incubated at 14°C for 1 hr. RNase was subsequently inactivated by the addition of 50 μg of proteinase K and 10 μl of 20% SDS and an incubation at 37°C for 15 min. Samples were phenol/chloroform extracted, ethanol precipitated, and resuspended in loading buffer containing 80% formamide for analysis on 5% denaturing polyacrylamide gel electrophoresis. A MspI digest of pBR322 was used as size markers. Gels were dried and bands visualized on Kodak XAR-5 film with intensifying screens.

The HLA-E antisense RNA template (Fig. 1A) was constructed by subcloning a 702-base pair (bp) PstI/XbaI fragment (positions 1427-2129 bp, ref. 3) containing 258 bases of α2 region (positions 1427–1685 bp) into the pSP6 vector

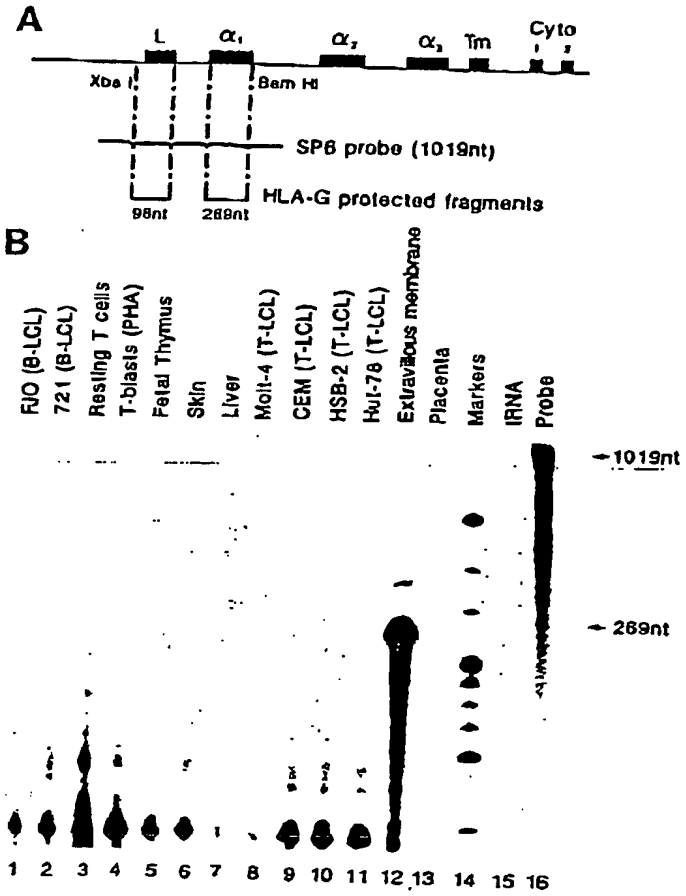

FIGURE 2 HLA-G RNA expression in human cells and tissues. (A) Diagram of the Sp6 antisense RNA probe used for detecting HLA-G transcript by RNase protection. Full-length antisense HLA-G RNA probe was 1019 nt in length. Protection by HLA-G mRNA yields two fragments 98 and 269 nt in length. (B) ^{32}P-labeled antisense probe was hybridized to 12 μg of RNA, digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis (lanes 1–13). Lane 15 depicts probe hybridized to tRNA alone. Arrows indicate positions of the full-length 1019-nt probe and the 269-nt HLA-G protected fragment.

according to standard methods [15]. The HLA-G antisense RNA template (Fig. 2A) was generated by subcloning a 1019-bp XbaI/BamHI fragment containing leader sequence and α1 region (positions 324–1343 bp, ref. 5) into the pSP6 vector.

RESULTS

Detection of HLA-E and HLA-G RNA. Analysis of one non-A, B, C HLA class I gene, HLA-F, expression in several different human cell and tissue types by RNase protection has been reported previously [4]. Analyses of various human cell lines and tissues for HLA-E and HLA-G expression by RNase protection are shown in Figs. 1 and 2, respectively. In agreement with the Northern blot data [3], HLA-E transcript was detectable in RNA from each cell line and tissue examined. The amount of HLA-E transcript detected varied between the different cell and tissue types (Fig. 1B). The highest level of HLA-E transcript was detected

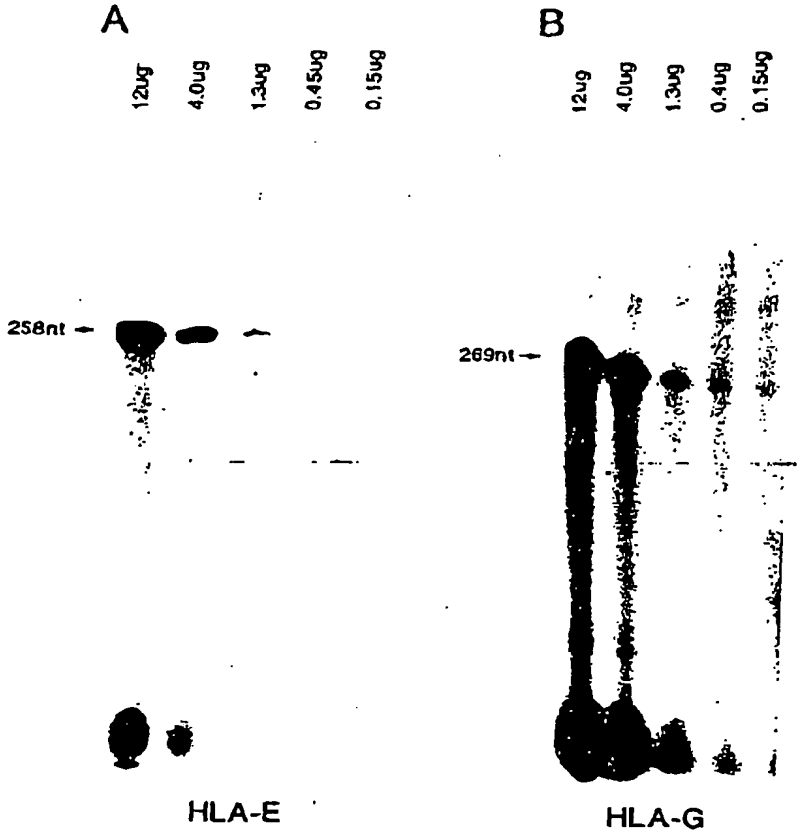

FIGURE 3 Sensitivity of the HLA-E and -G RNase protection assays. Sp6 antisense RNA probes for HLA-E (A) and HLA-G (B) were hybridized to serial dilutions of RNA from T cells (HLA-E), and term extravillous membrane (HLA-G), digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis. Arrows indicate the positions of the protected fragments for each gene.

in resting peripheral blood T lymphocytes. After activation of peripheral blood T lymphocytes by PHA, the amount of the HLA-E transcript decreased dramatically. An intermediate level of HLA-E transcript was detected in RNA from B-LCL FJO, B-LCL 721, fetal thymus (22nd week of gestation), CEM, HSB-2, and HUT-78. A low level of HLA-E transcript was detected in RNA from liver and Molt-4.

In contrast to HLA-E and -F [4], HLA-G transcript was detectable only in RNA isolated from term extravillous membrane (Fig. 2B), and not in RNA from all other cell and tissue types examined.

Detection limit of the RNase protection assays. To demonstrate the minimum amount of transcript from the HLA-E and -G genes detectable by the RNase protection assays, a serial dilution of RNA from the cell line or tissue expressing the highest level of HLA-E and HLA-G transcript was analyzed (Fig. 3A and B). For HLA-E, RNA from resting T cells was used. RNA from term extravillous membrane was used in the case of HLA-G. A series of threefold dilutions from 12 to 0.15 μg of cellular RNA were performed and each analyzed by RNase protection using the HLA-E and -G probes. After a period of exposure similar to that used for Figs. 1 and 2, HLA-E transcript was readily detectable

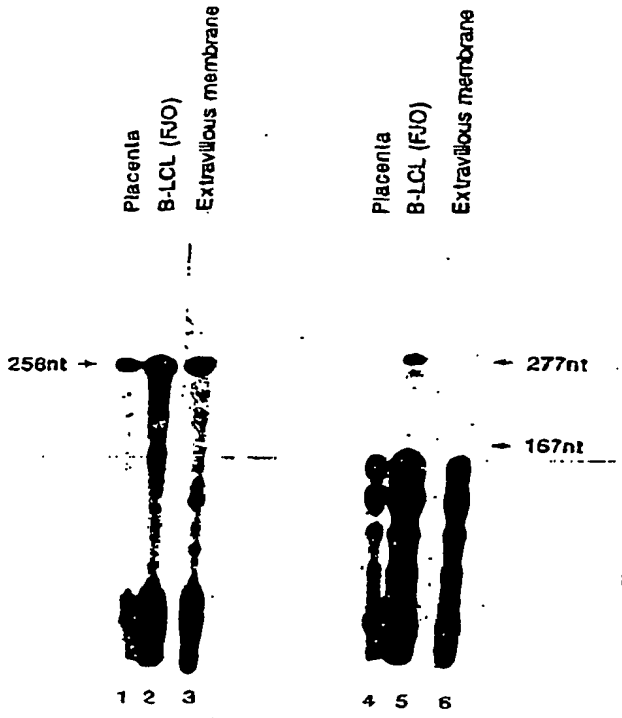

FIGURE 4 Detection of HLA-E and HLA-F transcripts in RNA from term placenta and term extravillous membrane. Full-length antisense RNA probes for HLA-E (lanes 1–3) and HLA-F (lanes 4–6) were hybridized to 12 μg of RNA, digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis. The antisense RNA probe used for detection of HLA-F transcript was as described previously [4]. Arrows indicate the positions of the protected fragments for each gene.

over a 10-fold range, and HLA-G transcript was detectable over a 26-fold range.

Expression of the non-A,B,C genes in extraembryonic tissues at different times of gestation. Since HLA-G transcript was found in RNA from extraembryonic tissue, placenta and term extravillous membrane were also examined for expression of the other two non-A,B,C class I genes, HLA-E and HLA-F. Figure 4 shows that RNA from both term placenta, lane 1, and term extravillous membrane, lane 3, protected an HLA-E-specific fragment of 258 nucleotides (nt). Furthermore, the level of HLA-E transcript found in RNA from term placenta and term extravillous membrane was comparable to that found in the B-LCL FJO (Fig. 4, lane 2). In contrast, RNA from term placenta and term extravillous membrane was unable to protect the HLA-F-specific fragment of 277 nt, lanes 4 and 6, respectively.

To determine if HLA-G and HLA-E expression in extraembryonic tissue changes during gestation, RNA from extraembryonic tissues at the 10th and 21st week of gestation was compared with RNA from term extraembryonic tissue for its ability to protect the HLA-G- and HLA-E-specific fragments. Figure 5 demonstrates that the ability of placental RNA to protect the HLA-G-specific 269-nt fragment varied during gestation. RNA from 10th-week placenta protected the 269-nt HLA-G fragment while 10th-week extravillous membrane RNA did not (Fig. 5, lanes 1 and 2). At the 21st week of gestation the 269-nt HLA-G fragment was protected by both placental and extravillous membrane RNA (Fig. 5, lanes 3 and 4). At term, as described previously, the HLA-G fragment was protected only by extravillous membrane RNA and not by placental RNA (Fig. 5, lanes 5 and 6; Fig. 2, lanes 12 and 13). Thus, the expression of HLA-G transcript

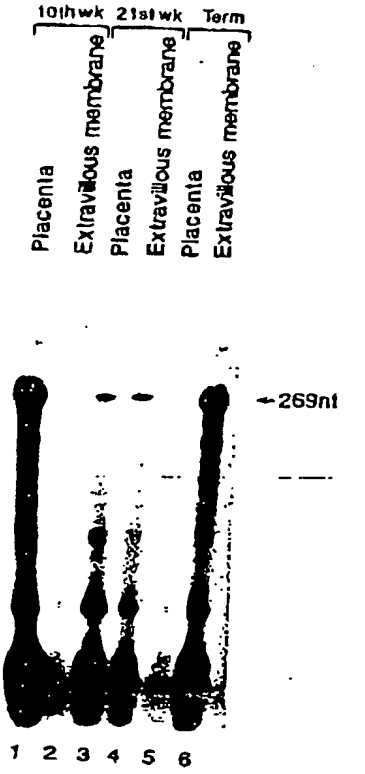

FIGURE 5 Detection of HLA-G transcript in RNA from placenta and extravillous membrane during gestation. Sp6 antisense RNA probe was hybridized to 12 μg of RNA, digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis. Arrow indicates position of the 269-nt HLA-G protected fragment.

shifts from the placenta early in gestation to the extravillous membrane late in gestation.

Figure 6 shows that at each time of gestation, RNA from both placenta and extravillous membrane was able to protect the 258-nt HLA-E-specific fragment. Thus, HLA-E transcripts are present in relatively high levels in both placental and extravillous membrane RNA and the pattern of HLA-E RNA expression in these tissues does not change dramatically during gestation.

DISCUSSION

In this study, a gene-specific and sensitive RNase protection method was used to examine the expression of the human non-HLA-A, -B, -C class I genes, HLA-E and HLA-G, in various tissues. This assay was able to demonstrate HLA-E expression in a variety of tissues obtained from many different individuals. In each case, a protected fragment of 258 nt was found. In case of HLA-G expression, extraembryonic tissues obtained from nine different individuals each demonstrated the presence of a protected fragment of 269 nt. Since the probes used for RNase protection assays were derived from the $\alpha 1$ region of HLA-G and $\alpha 2$ region of HLA-E, regions found to be highly polymorphic in HLA-A, -B, and -C, detection of protected fragments of identical size using RNA from different individuals strongly indicates that these regions of HLA-E and HLA-G are conserved in nucleotide sequence. Similarly, RNase protection analysis has demonstrated that the $\alpha 2$ region of HLA-F is also nonpolymorphic [4].

Table 1 summarizes the results presented in the previous study on HLA-F

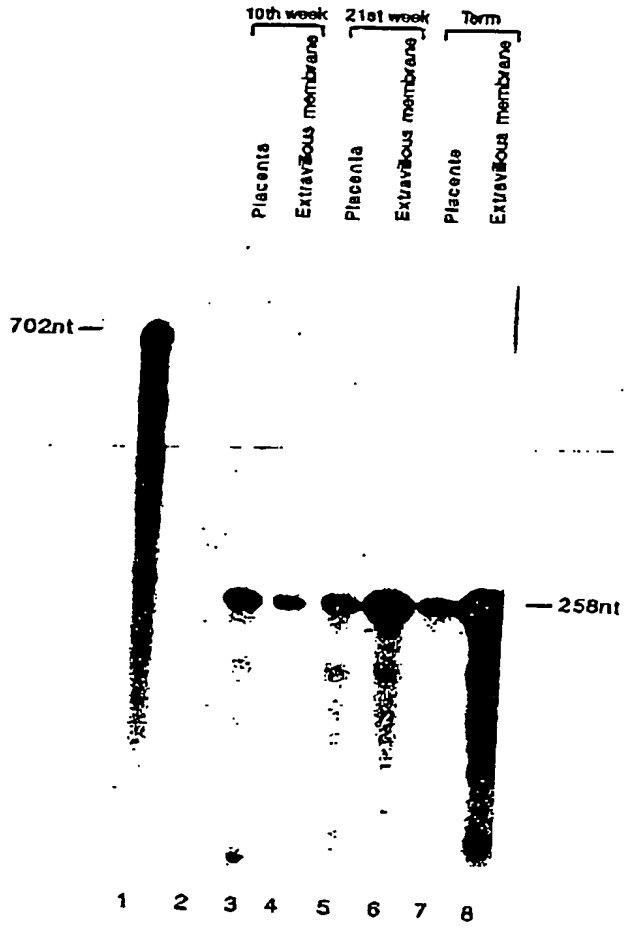

FIGURE 6 Detection of HLA-E RNA transcript in RNA from placenta and extravillous membrane during gestation. Sp6 antisense RNA probe was hybridized to 6 μg of RNA, digested with ribonucleases, and subjected to denaturing polyacrylamide gel electrophoresis (lanes 3–8). Arrows indicate positions of the 702-nt full-length antisense probe and the 258-nt HLA-E protected fragment.

expression [4] with those obtained here on the detection of HLA-E and HLA-G transcripts in a variety of human cell and tissue types. The pattern of expression found for HLA-E and HLA-F transcripts is, for the most part, very similar to that seen for the classical HLA class I genes, HLA-A, -B, and -C. Higher levels of HLA-E and HLA-F RNA were found in lymphoid cells as opposed to nonlymphoid cells. Moreover, within lymphoid tissues, mature T-cell lines expressed higher levels than immature T-cell lines. As noted previously [3], HLA-E RNA levels in T cells decreased upon their activation by PHA.

HLA-E and HLA-F transcripts were also detectable in RNA from nonlymphoid tissues. The previous study demonstrated a relatively low levels of HLA-F transcript in skin RNA and a very low level in RNA from liver [4]. In this study, HLA-E transcripts were detectable in RNA from all tissue and cell types examined, consistent with the previous Northern results [3]. RNA from liver had a very low amount of HLA-E transcript. However, RNA from three nonlymphoid tissues, skin, placenta, and extravillous membrane, contained relatively high levels of HLA-E transcript.

The present study demonstrates that, compared to other HLA class I genes, the HLA-G gene has a very restricted tissue pattern of expression. Of the tissue and cell types examined, detectable levels of HLA-G transcript were found only in RNA from placenta and extravillous membrane. Furthermore, the level of

TABLE 1 Expression of HLA-E, -F, and -G RNA in human cell lines and tissues

Cell/tissue	HLA-E	HLA-F	HLA-G
FJO (B-LCL)	++	++	−
721 (B-LCL)	++	++	−
Resting T cells	++++	++	−
T-blasts (PHA)	++	++	−
Fetal thymus	++	+	−
Skin	++	+	−
Liver	±	+/−	−
Molt-4 (T-LCL)	+	−	−
CEM (T-LCL)	++	−	−
HSB-2 (T-LCL)	++	+	−
Hut-78 (T-LCL)	++	+	−
Extravillous membrane, 1st trimester	+	−	−
Placenta, 1st trimester	+	−	++++
Extravillous membrane, 2nd trimester	++	+/−	++
Placenta, 2nd trimester	+	−	++
Extravillous membrane, term	++	+/−	++++
Placenta, term	+	−	−

HLA-G transcripts in placenta and extravillous membrane changes with time of gestation. At the earliest time of gestation examined, the 10th week, HLA-G transcript was found in RNA from placenta and not in RNA from extravillous membrane. At the 21st week of gestation both placental and extravillous membrane RNA contained HLA-G transcripts. At term, HLA-G transcripts were detectable only in RNA from the extravillous membrane.

An interesting point concerns the cellular sites of HLA-E and HLA-G expression within the placenta and extravillous membrane. By using cDNA probes and monoclonal antibodies, which were unable to distinguish between the products of HLA class I genes, both class I transcripts and proteins were detected in extravillous cytotrophoblasts at first trimester and at term [16–19]. However, in placental villous cytotrophoblasts at first trimester and at term only HLA class I mRNA was detected. Furthermore, HLA-G expression has been detected in chorionic cytotrophoblasts at term [20]. Recently, Kovats et al. [21] demonstrated that both HLA-G transcripts and proteins were detected in first-trimester villous cytotrophoblast, and were greatly reduced in the third-trimester villous cytotrophoblasts. From these data, we conclude that the placental cytotrophoblasts are the source of HLA-G RNA detected using RNase protection at first trimester (10th week of gestation). It is also likely that in term extravillous membrane, fetal cytotrophoblasts are the major sites of HLA-G expression.

Conclusions concerning the cellular site of HLA-E expression within extraembryonic tissue are less clear. HLA-E expression has been detected by Northern analysis [3] and RNase protection (this report) in a variety of cell types in addition to extraembryonic tissues. Thus, it is possible that HLA-E expression within extraembryonic tissue is not restricted to a single cell type. Term placental villous cytotrophoblasts are greatly reduced in number [22]. Since the level of HLA-E expression remains constant throughout gestation, its expression is not likely to

be restricted to fetal villous cytotrophoblasts. Elucidation of the exact cellular source(s) of HLA-E expression in extraembryonic tissue must await the development of specific reagents suitable for histologic studies.

Restriction of HLA-G expression to fetal extraembryonic tissue implies that HLA-G subserves a function critical to this tissue. Within the extraembryonic tissues, fetal cytotrophoblasts are exposed to the maternal decidua. It has been suggested that fetal trophoblasts function to shield the fetus from recognition by the maternal immune system [23]. Clearly, the lack of HLA-A,B,C expression by these cells is consistent with such a function [24]. Expression of the nonpolymorphic MHC class I molecule, HLA-G, should not result in fetal rejection by the maternal immune system, assuming the maternal immune system has been educated by HLA-G. Others have proposed that HLA-G may function to directly protect fetal cytotrophoblasts by activation of maternal suppressor cells or blockage of maternal cytotoxic cells [21].

HLA-G expression by fetal cytotrophoblasts may also function to enable the maternal immune system to survey these fetal cells, much as it does other maternal somatic cells. An immune surveillance capability would seem warranted since fetal trophoblast cells play a crucial role in the development of the embryo. Clinically, it is possible to have a placenta without an embryo, but it is not possible to have an embryo without trophoblasts [25]. The HLA-G primary structure is highly homologous to HLA-A, -B, and -C [5]. This indicates that the HLA-G protein molecule has a three-dimensional structure very similar to that of HLA-A2 [26]. Thus, HLA-G should be able to bind and present a peptide in a manner similar to HLA-A, -B, and -C molecules [27]. HLA-G on the surface of fetal cytotrophoblasts could afford a mechanism by which an antigen is presented to the maternal immune system, for example, in case of a viral infection, while keeping the fetal cells from being seen as foreign by the maternal immune system.

ACKNOWLEDGMENTS

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The Journal of Immunology

HLA-F Surface Expression on B Cell and Monocyte Cell Lines Is Partially Independent from Tapasin and Completely Independent from TAP[1]

Ni Lee and Daniel E. Geraghty[2]

In this study we examined HLA-F expression in normal cells and cell lines, with a particular focus on identifying cells that express surface protein. While HLA-F protein was expressed in a number of diverse tissues and cell lines, including bladder, skin, and liver cell lines, no surface expression could be detected in the majority of them. However, surface expression was observed on EBV-transformed lymphoblastoid cell lines and on some monocyte cell lines. Expression on B lymphoblastoid cell lines was observed, while no surface expression on normal B cells or on any peripheral blood lymphocytes could be detected. Surface expression correlated with the presence of a limited amount of endoglycosidase H (Endo H)-resistant HLA-F. However, clearly not all surface-expressed HLA-F was fully glycosylated. We further examined the requirement of HLA-F surface expression for functional TAP and tapasin molecules and identified a clear departure from the dependence shown by other class I molecules on TAP. In contrast, of the two surface glycosylation forms expressed, an Endo H-sensitive form was tapasin independent, while an Endo H-resistant form was clearly tapasin dependent. Finally, we tested whether HLA-F could be stabilized for surface expression without peptide by using the classical cold treatment for surface stabilization of empty class I. Of several cell lines tested, only MHC deletion mutant 721.221 demonstrated a typical class I phenotype, indicating that control of surface stabilization may have a genetic basis resident in the MHC. *The Journal of Immunology*, 2003, 171: 5264–5271.

The MHC in humans encodes a number of class I genes that serve fundamental roles in the acquired and innate immune responses. The classical class I proteins, named HLA-A, -B, and -C, bind and present peptide to CTLs and interact with receptors on NK cells to inhibit lytic activity or cytokine production (1–3). Intracellular transport and cell surface expression of class I proteins are dependent on the availability of peptides within the endoplasmic reticulum (4, 5). Three additional class I genes, HLA-E, -F, and -G, commonly referred to as nonclasssical or class Ib genes, are all highly homologous to the transplantation Ag genes. These gene products similarly associate with β_2-microglobulin (β_2-m)[3] and make up the remainder of the functional HLA class I gene family (6–8).

Polymorphism among HLA class I Ags has long been thought of as a hallmark of the functional diversity required of these molecules (9). While high levels of polymorphism in HLA class I have been maintained by overdominant selection (10), in contrast, the nonclassical class I molecules HLA-E, -F, and -G have been under a distinct selective pressure, exhibiting very low levels of allelic polymorphism. These low levels of allelic polymorphism are presumably reflected in their respective specialized functions. Indeed, HLA-G has long been thought to be restricted in allelic polymorphism due to its expression at the maternal-placental interface, possibly avoiding an alloresponse to paternal HLA-G. This restriction may limit the ability of HLA-G to bind peptide in the placenta and thus its ability to present Ag to T cells (11). In addition, HLA-G may assume a role interacting with NK inhibitory receptors (12, 13) and, alternatively or in addition, as a substitute to classical class I, acting through ILT2 or ILT4 to control inflammatory responses and cytotoxicity mediated by myelomonocytic cells (14, 15).

Progress understanding the biology of HLA-E has yielded definitive data about the function of this molecule. We previously showed that the availability of a nonamer peptide derived from certain HLA class I signal sequences is a necessary requirement for the stabilization of endogenous HLA-E expression on the surface of 721.221 cells (16). Knowing this, it was possible to implicate the CD94/NKG2A complex as an inhibitory receptor recognizing this class Ib molecule by using as target a .221 transfectant that selectively expressed surface HLA-E (17, 18). The crystal structure of HLA-E demonstrates that the specificity of leader peptide binding is a structurally defined intrinsic property of HLA-E (19). Of the two allelic forms of HLA-E, there were clear differences in the relative affinity for peptide of each heavy chain that correlated with and may be explained by differences between their thermal stabilities (20).

The function of HLA-F is unknown, but tissue- and cell-specific mRNA expression has been observed (21), and protein expression has largely correlated with that (22, 23). Two studies have reported that cell surface expression was not observed in any tissue or cell type studied, while a third demonstrated that HLA-F could be found on the surface of a subset of extravillous trophoblast cells that had invaded the maternal decidua (11). HLA-F may bind TAP, but unlike the classical human class I molecules, it was not detected at the cell surface regardless of the availability of TAP (22). Although no peptide or ligand binding to HLA-F has been described, through the use of HLA-F tetramer refolded with β_2-m, but without peptide ligand, an interaction with ILT2 and ILT4 was

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[3] Abbreviations used in this paper: β_2-m, β_2-microglobulin; Endo H, endoglycosidase H; B-LCL, B lymphoblastoid cell line.

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implicated (23). Whether this binding is indicative of a functional interaction with HLA-F is unclear, as similar binding has been observed with classical class I and HLA-G (14, 15).

In this study we used new mAbs reactive with HLA-F to study HLA-F expression in normal cells and cell lines. While HLA-F protein was expressed in a number of diverse tissues and cell lines, no surface expression was detected in the majority of them. However, surface expression was observed on EBV-transformed lymphoblastoid cell lines and on some, but not all, monocyte cell lines. This was true even though no surface expression on normal B cells or on any peripheral blood lymphocytes could be detected. Surface expression correlated with the presence of a limited amount of glycosylated HLA-F, although clearly not all the surface-expressed HLA-F was fully glycosylated. These studies further examined the requirement of HLA-F surface expression for functional TAP and tapasin molecules, identifying partial overlap and some clear departure from the dependence shown by other class I molecules. Finally, we tested one measure of the dependence of HLA-F to be stabilized for surface expression, finding further differences between HLA-F and other class I molecules in this regard. This latter feature may have a genetic basis resident in the MHC and independent of the HLA-F locus, as MHC deletion mutant cell line 721.221 demonstrated a more typical class I phenotype.

Materials and Methods

Monoclonal Ab production

HLA-F-specific Abs were generated by immunization of HLA-B27 transgenic mice with HLA-F inclusion body-derived heavy chain protein. The procedures used were essentially those described previously for anti-HLA-G and -E Abs (16, 24). Briefly, 100 μg of HLA-F protein in CFA was administrated in each immunization at 3-wk intervals. One month after the fifth immunization, there were three boosts every other day without adjuvant and mouse splenocytes that were fused with FOX-NY myeloma cells. Ab-secreting hybridomas were selected by ELISA against HLA-F, and cross-reactive Ab-producing hybridomas were eliminated by ELISA against recombinant HLA-E, -G, and -A2 proteins. Hybridomas from positive wells were subjected to two or three rounds of cloning by limited dilution. Cross-reactivity with other MHC class I was further examined by Western and FACS staining using .221 cells transfected with 12 distinct HLA-A, -B, and -C alleles. Subsequent Western and immunoprecipitation analysis from a variety of B lymphoblastoid cell lines (B-LCLs) (see below) further demonstrated specificity for HLA-F. In addition, PBL and LCL derived from the corresponding individual were obtained from the International Histocompatibility Working Group cell and gene bank (www.ihwg.org) and were tested in FACS and Western analyses for cross-reactivity with other HLA class I allotypes.

Cells and cell lines

Peripheral blood was obtained from healthy donors, and PBMC were isolated by Ficoll-Hypaque centrifugation. EBV transformation of PBMC was performed according to a standard protocol (25) using virus strain B95-8. The B-LCL lines 721.45.1, .134, .221, .221-B*27052, .221-C*0402, 2C2, .220, and .220+TPN (4, 16, 26, 27) were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM glutamine, and 1 mM sodium pyruvate. The amnion lines FL and WISH; bladder line J82; brain line Hs 683; colon line CCD-18 Co-5; intestine line HISM; kidney line 293; liver line Chang Liver; lymphoblast lines RPMI7666 and MOLT-3; lymphoma lines U937 and 8E5; leukemia line K562; lung line Calu; placenta lines JEG, BeWo, and 3A-SubE; skin lines BUD-8 and A-431; thymus line Hs67; pancreas line AsPC-1; and monocyte lines KMA, 90196B, and MD were all obtained from America Type Culture Collection (Manassas, VA) and cultured according to the product information sheet.

Expression of recombinant HLA-F protein

HLA-F plasmid was constructed, and recombinant protein was purified as described previously for the production of recombinant HLA-E (20). Briefly, the full-length of HLA-F cDNA was prepared from RNA using .221 cells by RT-PCR. DNA coding for a GlySer linker and a BirA substrate peptide (28) was fused to a cDNA encoding 276 aa of the HLA-F heavy chains (excluding the signal sequence) by PCR with the 5′ primer CGCGCGAATTCAGGAGGAATTTAAAATGGGCTCCCACTCCTTG and the 3′ primer GCGCAAGCTTTTAACGATGATTCCACACCATTTTCTGTGCATCCAGAATATGATGCAGGGATCCCTGCTCCCATCTCAGGATGAGGGGCTGG. The underlines contain *Eco*RI and *Hin*dIII restriction sites, respectively. PCR products were ligated into pHN1$^+$ vector and expressed in *Escherichia coli* strain UBS. β_2-m in pHN1$^+$ was provided by D. C. Wiley (Harvard University, Cambridge, MA) and expressed in *E. coli* strain XA90. Both heavy and light chain inclusion bodies were isolated from cell pellets and washed repeatedly in detergent, and the resulting protein was solubilized in urea.

Immunofluorescence staining and FACS analysis

mAbs 3D11, 4A11, and 3D12 were employed in indirect immunofluorescence staining as previously described (16). Briefly, cells were preincubated with saturating concentrations of primary Abs, followed by washing and labeling with FITC-conjugated goat F(ab′)$_2$ anti-mouse Ig (BioSource, Camarillo, CA). Samples were analyzed on a FACScan cytometer (BD Biosciences, Mountain View, CA).

Cell surface biotinylation, immunoprecipitation, and endoglycosidase H (Endo H) digestion

For cell surface labeling, PBS-washed cells were biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, IL; 300 μg/ml) for 30 min at 4°C. After being washed twice with PBS, cells were treated with 50 mM glycine at 4°C for 5 min and washed extensively. Cells were lysed at 20 × 10^6 cells/ml of lysis buffer containing 10 mM Tris (pH 7.8), 140 mM NaCl, 1% Triton X-100, 200 μM PMSF, 10 μg/ml of papstatin, and 14 μg/ml of aprotinin. After incubation on ice for 1 h, the lysate was centrifuged at 11,000 × *g* for 20 min, and the supernatant was collected. Cell surface proteins were precipitated with 50% streptavidin-agarose (Pierce) overnight at 4°C. The streptavidin-agarose beads were separated from the cell lysate by centrifugation; washed five times in 10 mM Tris-HCl (pH 7.5), 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.1% SDS, and 300 mM NaCl and once in 0.5% Nonidet P-40 in PBS; resuspended in Endo H digestion buffer (100 mM sodium citrate/phosphate (pH 5.5), 0.1% SDS, and 50 mM 2-ME); and boiled for 5 min.

The remaining cell lysate was precleaned extensively with Ig control Ab and protein A-Sepharose. Following centrifugation, the supernatant was adjusted to 0.2% SDS and 1 μg/ml BSA, and W6/32 at 5 μg/ml (final concentration) was added. After continuous mixing at 4°C for 1 h, 50% protein A-Sepharose was added at 150 μl/ml and incubated at 4°C for 2 h. The immune complex was washed twice in PBS containing 0.1% SDS and 0.5% BSA; three times with buffer containing 10 mM Na_2HPO_4, 0.5% Nonidet P-40, 0.5% BSA, and 0.5 M NaCl; and once in 0.5% Nonidet P-40 in PBS; resuspended in Endo H digestion buffer as described above; and boiled for 5 min. Endo H (Glyko, Novato, CA) digestion was conducted according to the manufacturer's suggestion. In some experiments cells were not surface biotinylated; instead, total cell lysate was immunoprecipitated with W6/32 and subsequently digested with Endo H as described above. Samples derived from 5 × 10^5 cells were separated in 11% SDS-PAGE gel and examined by Western blot analysis.

Western blotting and immunodetection

Cells were lysed, and total cell lysate was separated on a 10% SDS-PAGE gel as described previously (16). Briefly, cells were lysed in 10 mM Tris (pH 7.5), 140 mM NaCl, 1% Triton X-100, 0.1 mM PMSF, 10 μg/ml pepstatin, and 14 μg/ml aprotinin at 20 × 10^6/ml. Lysate from 45 × 10^4 cells was loaded onto each well, and proteins were transferred to nitrocellulose membrane (S and S; Schleicher & Schuell, Keene, NH). HLA-F protein was detected by mAb 3D11, followed by HRP-labeled goat anti-mouse Igs (BioSource) at 1/5000 dilution and visualized with an ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL). For analysis of cell surface and internal proteins, HLA-E and -F proteins were detected by mAbs 7G3 and 3D11, respectively.

Results

HLA-F Abs and protein expression

Arguably, the most effective strategy toward a complete analysis of the nonclassical Ags includes the development of specific mAbs. We had previously succeeded in developing monoclonals against HLA-E and -F and soluble and membrane-bound forms of HLA-G (11, 16, 24) and have continued that progress here. To obtain sufficient HLA-F Ag for immunization, we used recombinant HLA-F synthesized in *E. coli* as described in *Materials and Methods*. Our Ab screening method relied on an ELISA using

W6/32 as capture Ab and refolded recombinant HLA-F as Ag. From this screening we were able to identify three reagents that showed specificity when tested in Western analysis and immunoprecipitation from LCL .221. These cells expressed only HLA-E and -F (29), and it was possible to distinguish the HLA-F IEF pattern given the known and characterized HLA-E pattern (16). The m.w. of HLA-F is lower than those of other classical class I and HLA-E due to the absence of exon 7 in the mature transcript (21), allowing HLA-F to be distinguished according to its m.w.. Specific reactivity with HLA-F was confirmed by isoelectric focusing and by m.w. in immunoprecipitation and Western analyses using normal LCL.

The specificity of these reagents for HLA-F and a definition of cross-reactivity with other class I was further tested in FACS experiments with B-LCLs and corresponding PBL derived from the same donor. In an examination of 20 individuals selected to represent a diverse collection of HLA class I allotypes, PBL showed no reactivity with Abs 3D11 or 5E8. Ab 4A11 did, however, appear to react with a subset of the HLA-Cw4 alleles (specifically with C*0401, C*0404, and C*0405, but not with C*0403). This cross-reactivity was confirmed using .221 transfected with HLA-C*0401. These cross-reactive Cw4 alleles share with HLA-F a glutamic acid residue at position 49 in the α1 domain not found in any other class I A, B, or C allele. In addition, Western analysis of these cell lines using 3D11 or 4A11 (5E8 did not work in Western analysis) corroborated the FACS results, as we were able to distinguish HLA-F from classical class I due to its lower m.w.. It should be noted that one of these Abs was used (3D11), and two were described (3D11 and 4A11) in a recent study (11), while 5E8 is first reported here.

In an attempt to gain an initial insight toward HLA-F protein expression in normal tissue, we first analyzed a collection of cells and cell lines obtained from American Type Culture Collection (Manassas, VA) and derived from a diverse set of tissues by Western blotting. Fig. 1 shows a representative view of this analysis,

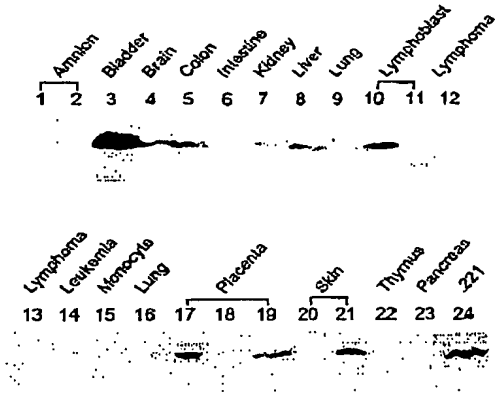

FIGURE 1. HLA-F expression is detected in specific tissue-derived cell lines. Shown are results of Western blot analysis of cell lysates from representative human cell lines detected with anti-HLA-F reagent 3D11. The tissue from which the lines were originally derived is indicated above the lanes. The numbering above each lane corresponds to cell lines as designated by American Type Culture Collection as follows: 1) FL; 2) WISH; 3) J82 transitional cell carcinoma; 4) Hs 683 glioma; 5) CCD-18 Co-5; 6) HISM; 7) 293 transformed by Ad5; 8) Chang Liver; 9) CCD-11Lu; 10) RPMI 7666 transformed by EBV; 11) MOLT-3 T cell leukemia; 12) U937 T cell lymphoma; 13) 8E5 T lymphoblastoid; 14) K562 chronic myelogenous leukemia; 15) THP1 acute monocytic leukemia; 16) Calu adenocarcinoma; 17) JEG choriocarcinoma; 18) BeWo choriocarcinoma; 19) 3A-subE SV40 transformed; 20) BUD-8; 21) A-431 carcinoma; 22) Hs67; 23) AsPC-1 adenocarcinoma; and 24) positive control 721.221.

demonstrating tissue-specific protein expression essentially in line with that previously described for mRNA expression (21). Some of these cells were derived directly from the tissue of origin and constitute lines with a finite life span, while others represent tumor lines derived from the cited tissue (details provided in *Materials and Methods* and from American Type Culture Collection). Some differences from other studies were apparent, as exemplified by skin cells, which were found to be positive in our study (Fig. 1, *lanes 20* and *21*) (22). We do not have an explanation for these discrepancies, although they could be cell line specific. The cells that tested positive were all further examined for surface expression of HLA-F using FACS analysis with labeled Abs 3D11 and 4A11. HLA-F could not be detected on the surface of any of the cells analyzed in Fig. 1 regardless of the apparent level of protein detected via Western analysis, with the notable exception of B-LCL RPMI 7666, which showed positive staining in FACS analysis (data not shown).

HLA-F is surface expressed on B cell and monocyte cell lines

It was reported that HLA-F, while expressed in B cells and B cell lines, was not surface expressed on either cell type (22, 23). However, because our initial survey of cells had shown positive FACS staining with a B-LCL, and since the previous studies had been performed using different Abs, we decided to carry out a comparison of normal peripheral B cells and EBV-transformed B cell lines derived from the same donor using the Abs developed in our laboratory. In this regard it is noteworthy that the two Abs used here (3D11 and 4A11) have distinct and nonoverlapping epitopes (data not shown), making the pair an effective combination toward confirming our results.

Our initial Western analysis demonstrated that both cell types expressed HLA-F, in agreement with the above-mentioned studies. However, while we did not find HLA-F expressed on the surface of peripheral B cells, we did find surface expression on all corresponding B-LCLs (Fig. 2). Although levels varied depending on the LCL examined, all B-LCL expressed detectable surface HLA-F at levels similar to HLA-E. To examine whether surface HLA-F was glycosylated in a manner similar to other class I, cells were lysed, class I protein was precipitated with pan class I reagent W6/32, and the resultant material was treated with Endo H (Fig. 2, *lower panel*). For MHC class I Endo H resistance is indicative of complex formation and transport through the Golgi to the cell surface (30). This analysis did indeed show a correlation between surface expression and the presence of some Endo H-resistant material consistent with that found for other class I, including HLA-E. This result was consistent with the FACS analysis, which demonstrated surface HLA-F expression. However, it should be noted that a substantial portion of HLA-F protein detected in this experiment was of the high mannose hybrid-type form (Endo H-sensitive). This was true both because this experiment examined total protein and because significant amounts of the Endo H-sensitive form are surface expressed (see below).

In a search for other cell types that might express surface HLA-F, we screened several lines using FACS analysis and anti-HLA-F reagents 3D11 and 4A11 in addition to those described above. This search yielded three cell lines, each of which had its origins as a monocyte-derived cell line and expressed surface HLA-F at levels similar to that seen for HLA-E (Fig. 3*A*). In these cells, surface HLA-F was examined separately from cytoplasmic HLA-F by cell surface biotinylation and immunoprecipitation with streptavidin, showing that some, but not all, surface HLA-F was in an Endo H-resistant form. This was in contrast to HLA-E protein, where essentially all surface protein was converted into an Endo H-resistant form, demonstrating the specificity of surface labeling

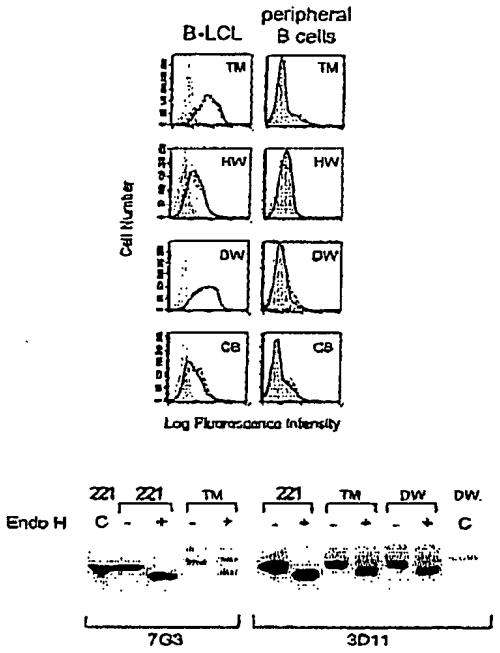

FIGURE 2. HLA-F surface expression is detected on EBV-transformed B cells, but not on peripheral B cells. *A*, FACS analysis of HLA-F expression in B-LCL cells (*left column*) and their counterpart peripheral B cells (*right column*) in pairs derived from each of four individuals was performed using the HLA-F-specific mAbs 4A11 (solid line) and 3D11 (dashed line). HLA-F expression was examined with 3D12 (dotted line). Shaded profiles were cells stained with isotype-matched control Ab. Initials identifying the individuals from which the cells were derived are present within the respective FACS profiles. *B*, Western blot analysis demonstrating that a portion of the HLA-F protein in normal B-LCLs, but not the class I-deficient line .221, is Endo H resistant. LCLs .221, TM, and DW LCLs were lysed; class I protein was precipitated with mAb W6/32, treated (+) or untreated (−) with Endo H; and SDS-PAGE was performed. The Western blot on the *left* was probed with anti-HLA-F mAb 7G3 as indicated to demonstrate the control for Endo H digestion, and on the *right*, material from the same LCLs was similarly examined for Endo H-resistant HLA-F material using anti-HLA-F mAb 3D11 as indicated beneath the blot. Undigested material at the *far left* and *right lanes* was added for local size comparison.

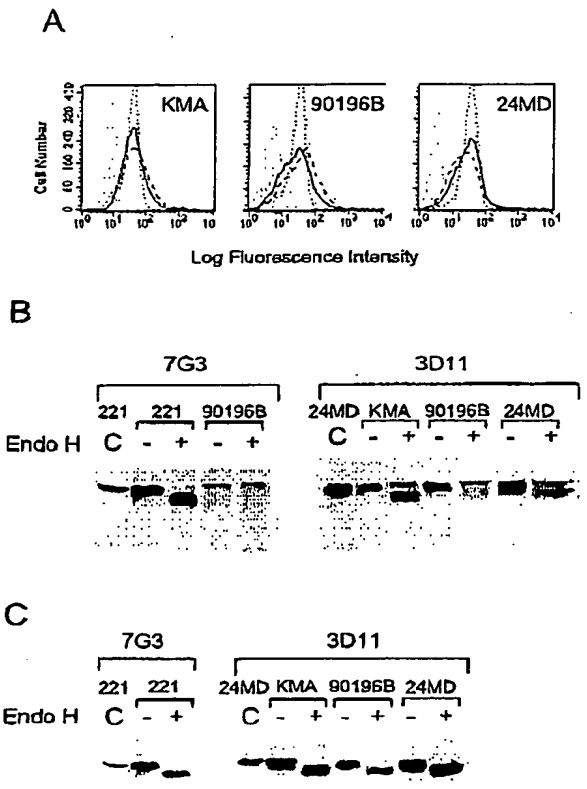

FIGURE 3. HLA-F is expressed on the cell surface of monocyte cell lines, and a portion of the surface complex is Endo H resistant. *A*, Monocyte cells were analyzed for surface expression of HLA-F by FACS analysis using mAb 4A11 (solid lines) and 3D11 (dashed lines). Shaded histograms show Ig isotype-matched control stainings. *B* and *C*, Cell surface proteins were biotinylated with sulfo-NHS-LC-biotin as described in *Materials and Methods*. Cells were then lysed and immunoprecipitated with streptavidin-agarose beads. The avidin-bound immunocomplexes (*B*) and the remaining lysates (*C*) were separated and subsequently treated (+) or untreated (−) with Endo H, separated in SDS-PAGE, and analyzed by Western blot analysis. HLA-F proteins were detected on a subset of the cell lines using mAb 7G3 (*left*) to demonstrate the control for Endo H digestion. HLA-F protein was detected using mAb 3D11 (*right*) on the same material.

(Fig. 3*B*). All the cytoplasmic HLA-F and HLA-E protein was in an Endo H-sensitive form, as expected for class I that has not transported through the Golgi (Fig. 3*C*).

HLA-F surface expression is independent of TAP, but partially dependent on tapasin

Given that HLA-F resembled other class I proteins with regard to surface expression and glycosylation, at least on B-LCL and monocyte cell lines, we undertook a comprehensive examination of HLA-F expression on the mutant cell lines that are deficient in normal TAP and tapasin function (4, 5). These cells expressed endogenous HLA-F, and therefore it was possible to examine them directly using the Abs described in this report. In addition, they expressed endogenous HLA-E and were used to examine HLA-E in previous studies (16), providing a useful control for the analysis of HLA-F. Whereas HLA-E showed the expected lack of surface expression in TAP and tapasin-negative cells, HLA-F surface expression was clearly unperturbed on the TAP-negative .134 mutant and was only moderately lowered in the tapasin mutant .220 (Fig. 4*A*). Indeed, there was no difference between HLA-F expression on parent line 45.1, line .134, or the TAP-restored line 2C2. It was further apparent that, as expected, all *N*-glycosylation of HLA-E in mutant cells was in the high mannose hybrid-type oligosaccharide (Fig. 4*B*), and the complexity of glycosylation was restored when TAP was restored in 2C2 cells. However, again in contrast, the glycosylation levels of HLA-F were unchanged in the TAP-negative line .134 regardless of whether total cellular protein or surface protein was examined (Fig. 4, *B* and *C*).

HLA-F expression did not appear to be independent of tapasin, because a difference in surface HLA-F was apparent when .220 was compared with the parent line 45.1, the TAP-negative line .134, or the TAP-restored line 2C2, and the reduced surface levels of HLA-F were restored in a tapasin transfectant of .220 (Fig. 4*A*). In this case the surface HLA-F lost was apparently all of the complex-type or Endo H-resistant glycosylation form. Further, this Endo H-resistant form was also restored in the tapasin transfectant of .220 (Fig. 4, *B* and *C*). All the material in .220 cells was sensitive to Endo H regardless of whether total protein or surface-labeled protein was examined (Fig. 4, *B* and *C*). However, despite the fact that all the HLA-F was the hybrid-type oligosaccharide, as

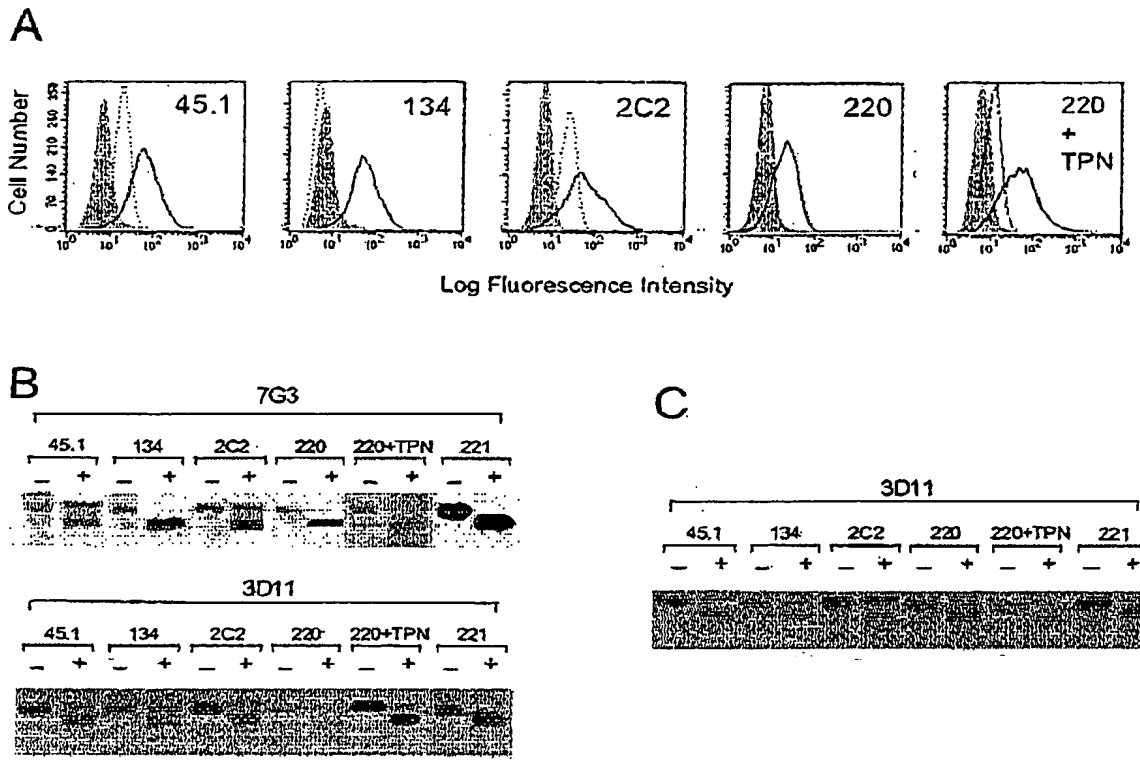

FIGURE 4. Cell surface expression of HLA-F on B-LCL is independent of the presence of a functional TAP protein and is partially independent of the tapasin molecule. *A*, FACS analysis of surface expression of HLA-F in the TAP-negative mutant .134 and LCL 2C2, the TAP-positive reconstitution of .134 (4), the tapasin-deficient cell line .220, and the tapasin-restored transfectant of .220 (.220+TPN) (27) using mAb 4A11 (solid lines). Parent LCL 45.1 (TAP and tapasin positive) is included for comparison. HLA-E surface expression, which is dependent on TAP and tapasin (16), was detected using mAb 3D12 (dotted lines) for comparison. Shaded histograms show staining of Ig isotype-matched control Ab. *B*, Western analysis of untreated (−) and Endo H-treated (+), W6/32-precipitated protein using anti-HLA-E reagent 7G3 and anti-HLA-F reagent 3D11. The same cell lines as in *A* were examined, with the addition of 721.221 to control for Endo H digestion of sensitive protein. *C*, Surface protein labeled with biotin and precipitated with avidin-coated beads was run untreated (−) or was subjected to Endo H (+) for each of the cell lines described in *B* and was detected with anti-HLA-F reagent 3D11 in Western analysis.

found for HLA-E, it should be clearly noted that this phenotype was distinct from HLA-E, because some HLA-F protein was still expressed on the surface. This result raised the possibility that on the cell surface the Endo H-resistant form of HLA-F was associated with β_2m, while the sensitive form was not. It was not possible to distinguish the surface and intracellular forms of HLA-F in these cells; however, it may be noteworthy that the β_2m-negative B cell line Daudi was devoid of surface HLA-F, although Daudi transfected with β_2m was also lacking surface expression (data not shown).

Stabilization of HLA-F surface protein is distinct from other class I

One feature of class I protein that is related to its ability to bind peptide and a direct measure of its stability without bound ligand is the stabilization of surface MHC class I by lower temperature (30). When TAP-negative human or murine mutant cell lines are cultured at reduced temperature (19–33°C), assembly of heavy chain and β_2-m is promoted and results in a high level of cell surface expression of MHC heavy chain/β_2-m complexes that do not present endogenous Ags and are labile at 37°C. This feature is common to all class I proteins, including HLA-E, and therefore provides a useful point to compare HLA-F expression with other class I proteins. Further, this test is one measure of whether HLA-F is lacking peptide in cells that express endogenous protein that is not surface expressed. In the present study we grouped cell types into three classes that expressed HLA-F protein, lines J82 or A431 that expressed significant levels of HLA-F intracellularly, but not on the surface; monocyte-related cell lines; and normal B-LCLs, both of the latter expressing HLA-F on the surface. When these cells were examined by incubation at 26°C, so-called cold treatment, it was apparent that HLA-F expression was unaffected in every case (Fig. 5). In no case did the level of HLA-F surface protein increase, and indeed, in some cases there was a slight decrease in expression. This was true despite the fact that HLA-E could consistently be stabilized and surface expression levels increased on these cells in precisely the same experiment, demonstrating the effectiveness of the treatment to stabilize class I.

During a survey of several cell types for HLA-F surface expression and stabilization, we found one exception that behaved consistently with other class I proteins. Deletion mutant line .221 showed a clear and relatively strong increase in surface HLA-F upon cold treatment, while the parent line .144 (hemizygous MHC and deficient in HLA-A and surrounding sequences) (31) and the grandparent line 45.1 (hemizygous MHC) showed no change upon similar treatment (Fig. 6). Again, an examination of HLA-E levels in the same experiment controlled for the effectiveness of the cold treatment. It is interesting to note that .221 is missing the same sequences surrounding HLA-A as .144, but is further missing, at the least, an extended portion of the MHC around and including the HLA-B and -C loci. Given transfectants of .221 that express the HLA-B and -C loci, we were able to rule out that this phenotype was not simply due to the absence of either of these two class I genes (as was the case with HLA-E) (16). No differences from .221

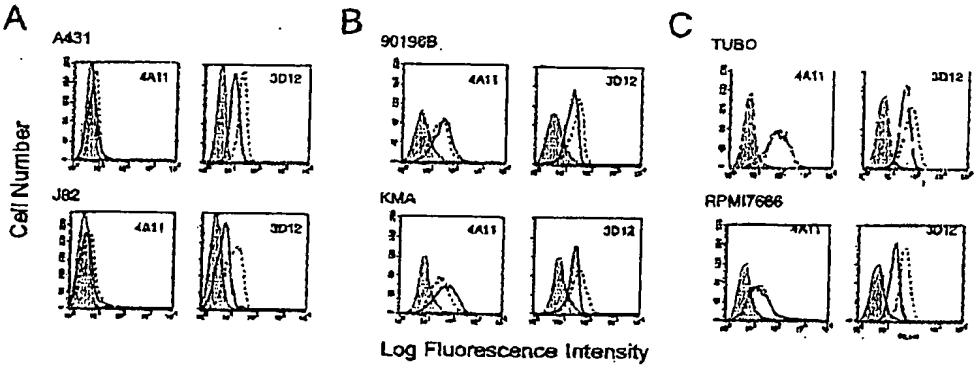

FIGURE 5. HLA-F surface expression is down-regulated or unaffected by cold treatment in cells expressing the protein. Detection of HLA-F on the surface of cells by flow cytometric analysis was performed using mAbs 4A11 and 3D11. HLA-E was detected using specific mAb 3D12 for comparison and as a control for temperature treatment, as previous studies have shown increased surface expression of HLA-E upon cold treatment (16, 18). Three types of cells were examined after normal growth conditions at 37°C and after treatment at 25°C for 24 h. *A*, Monocyte cell lines that express endogenous HLA-F but lacked surface expression (Fig. 1); *B*, monocyte cell lines that express surface HLA-F (Fig. 3); *C*, B-LCLs. Above and to the *left* of each quartet of FACS profiles is indicated the cell name, and within each profile is indicated the Ab used for staining. Cells incubated at 37°C (solid lines) and after treatment at 25°C for 24 h (dashed lines) were stained with the indicated mAbs. Overlapping gray profiles and dotted lines are the 37 and 25°C treated cells stained with Ig isotype-matched control mAbs, respectively.

were observed when .221-B*27052 or .221-C*0402 transfectants were tested for surface stabilization of HLA-F (Fig. 6).

Discussion

We have examined the expression of HLA-F protein with a particular focus on searching for surface expression and understanding the mechanisms regulating its cell surface expression. To accomplish this we generated mAbs that specifically react with HLA-F and that complement one another in their reactivity. In a survey of tissues and cell lines, we found that HLA-F was expressed intracellularly in a number of cell types, but was not transported to the surface in most of these. The two exceptions, EBV-transformed B cell lines and monocyte-derived cell lines, expressed HLA-F surface protein at levels comparable to HLA-E. Whereas none of the HLA-F protein expressed in deletion mutant LCL 721.221 was converted to the Endo H-resistant form, only a portion of the surface HLA-F on normal LCL was glycosylated in a manner typical of peptide-bound HLA class I. This was also true of the monocyte-derived cell lines, where some of the surface-expressed HLA-F was Endo H resistant, indicating a typical transport to the cell surface. These two cell types constituted the first examples of cell lines where HLA-F was surface expressed.

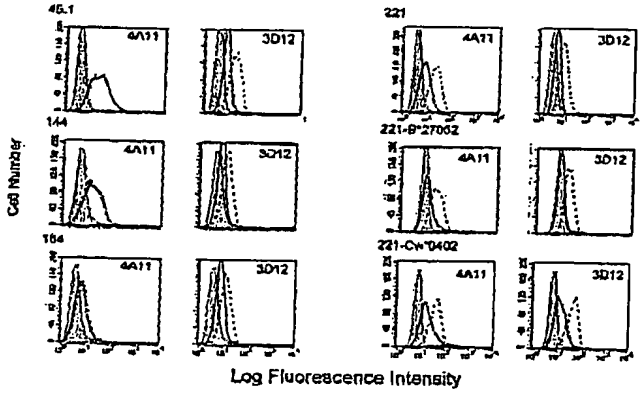

FIGURE 6. Class I deletion mutant LCL .221 shows exceptional expression of HLA-F after cold treatment. Detection of HLA-F on the surface of cells by flow cytometric analysis was performed using mAb 4A11. HLA-E was detected on the same cells using specific mAb 3D12 as a control for temperature treatment. The parent line LCL 45.1 is hemizygous for the MHC and primary offspring, and deletion mutant .144 (missing HLA-A and surrounding region) is a precursor of .221 (missing HLA-A, HLA-B, HLA-C, and some surrounding sequences). Class I transfectants of .221 expressing HLA-B*27052 and HLA-C*0402 were described previously (16). Cells incubated at 37°C (solid lines) and after treatment at 25°C for 24 h (dashed lines) were stained with the indicated mAbs. Overlapping gray profiles and dotted lines are the 37 and 25°C treated cells stained with Ig isotype-matched control mAbs, respectively.

HLA-F mRNA expression was examined in the original descriptions of the gene (21, 32), and the results found here agree with those with respect to the differential expression of HLA-F in tissues and cells. In general, HLA-F was expressed in B cell lines and was not present in T cell lines. Our results were mostly consistent with two other studies that examined HLA-F protein expression (22, 23), but differed notably with respect to surface expression on B cell lines, while neither examined protein expression on the three monocyte-derived cell lines reported here. However, at least three lines of evidence are consistent with the findings of surface expression we report here. First, the three distinct anti-HLA-F Abs we used gave consistent results in FACS analysis, and two of these, 3D11 and 4A11, gave consistent results in Western analysis (the third was not useful in Western analysis). In addition, we know that 3D11 and 4A11 react with distinct epitopes on HLA-F (D. E. Geraghty, unpublished observations), further increasing the confidence in these results. A second line of evidence supporting our conclusions is the observation of Endo H-resistant HLA-F protein coincident with surface expression on B cell and monocyte cell lines, and that this glycosylated form was restricted to the cell surface (Figs. 3 and 4). The loss of Endo H-resistant material coincident with decreased relative surface levels on mutant line .220 compared with its parent lines presents a third piece of supporting evidence for surface expression of HLA-F on B cell lines.

While some HLA-F surface protein showed glycosylation patterns consistent with peptide loading and transport through the Golgi, in B-LCLs there was apparently no dependence on TAP for surface expression. No decrease in HLA-F surface expression was apparent in the TAP-negative LCL .134 compared with either the parent line 45.1 or the TAP-restored derivative line 2C2. Also, Endo H-resistant protein was present in all three lines essentially

as observed in normal LCLs. It was shown that HLA-F associates physically with TAP, which indicated that it may depend on TAP for peptide binding (22, 23). This finding was surprising in light of the observation that residues 116 and 156 might play a role in MHC class I association with TAP (33), while both those residues are substantially altered in the HLA-F sequence (21). An explanation may be indicated by other evidence of one residue in the α2 domain of HLA-A2. Residues in the α3 domain have been shown to be involved in this association (34–36), and these positions are conserved in HLA-F. Regardless of which interpretation is relevant, the significance of TAP and HLA-F association is now in question, and the physical observation of this association may only rely on conserved residues and not be functionally relevant. There is no a priori reason to believe that association of class I with TAP necessarily indicates a corresponding dependence on TAP for peptide loading. Arguing from the logical contrary, soluble HLA-G does not associate with TAP, yet it depends on TAP for peptide loading (24).

While no dependence on TAP was observed at least in LCL, it was clear that there was some level of tapasin dependence for surface expression, as overall surface levels were reduced, and all Endo H-resistant material was absent in tapasin-negative .220. This could indicate that of the two forms of HLA-F on the surface, the Endo H-resistant form is peptide dependent in a manner typical of MHC class I. By analogy to other HLA class Ib proteins, there are two possibilities: 1) HLA-F binds a broad array of peptides similar to HLA-G (24); or 2) HLA-F binds a restricted set more in analogy to HLA-E (16). In our survey of a large number of cell lines for HLA-F surface expression, it was clear that while several cell types expressed HLA-F intracellularly, few expressed it on the surface. This could indicate that the specific peptide(s) required for HLA-F surface expression is restricted to a relatively small set of peptides. Such a restricted peptide-binding site has been proposed for HLA-F based on modeling the structure on other HLA class I (37).

Beyond the novel observation that HLA-F is surface expressed on certain cell types is the compelling question of whether this expression is dependent upon peptide or other ligand for complex stabilization as with other class I proteins. The fact that surface expression of HLA-F is TAP independent does not by itself contradict the potential for peptide binding. Several examples of the efficient assembly of class I and peptide in the absence of TAP have been described for peptides that are targeted into the endoplasmic reticulum in TAP-independent ways as long as tapasin is present (38, 39). There are many sources of TAP-independent peptides, and nonpeptide ligands are certainly not ruled out (39). Indeed, then, the ability of HLA-F to bind peptide is supported by the absence of complex-type HLA-F on tapasin-negative cells, so that at least this portion of the HLA-F surface protein might be complexed with peptide.

It was apparent that in both LCL and monocyte cell lines, two distinct forms of HLA-F were present on the surface: a high mannose hybrid-type form and a form with complex-type *N*-glycosylation. Although less common, examples of high mannose glycan-bearing proteins on the cell surface have been reported (e.g., CD2 (40) and the ME20 Ag (41)), and of the two glycosylation sites present on the HLA-DRA chain, one is high mannose and one is complex type on the surface protein (42). A rationalization for this difference is that the three-dimensional structure of the protein may dictate the accessibility of the high mannose sugars for further glycosylation (43). If this is true, then the finding of two distinct glycosylation forms of HLA-F might suggest that the protein is present in two distinct conformations. Since glycosylation is presumably stabilizing the glycoprotein structures (44), a consequence may be a differential in the half-life of the two forms of HLA-F.

Post-translational glycosylation is critical for the biological function of many proteins. The distinct HLA-F glycosylation patterns observed naturally raise the question of whether the two forms of HLA-F have distinct functions. Examples of both similar and dissimilar functions have been reported. High mannose structures on soluble CD154 compared with complex-type sCD154 glycoprotein showed no difference in complex formation, ligand binding, or function (45). However, changes in the glycosylation of CD44 did appear to have important regulatory effects on CD44 function (46), and the extent of glycosylation of the human prostacyclin receptor may be important for ligand binding and signal transduction (47). Glycosylation of MHC class I is not required for recognition by B or T cells, and at least for some of the inhibitory specificities examined, oligosaccharide is not necessary for activity of MHC class I and NK cell inhibitory receptors (48). However, intracellular trafficking appears to be significantly affected by changes in glycosylation or the lack thereof, as mutant class I heavy chains that lack glycosylation sites show inefficient transit to the cell surface. Possibly relevant to HLA-F, high mannose-type glycans, once phosphorylated, can interact with mannose-6-phosphate receptors to assist trafficking to the lysosomal compartment (49), speculatively in the example of HLA-F, for peptide loading there. In addition, CD1 days, an MHC complex class I-like protein, can be surface expressed in vivo in two forms, one Endo H-sensitive and one Endo H-resistant (50, 51). Further, CD1 days proceeds through both intrinsic and extrinsic pathways to complex with Ags, the former through the secretory pathway, and the latter where CD1 days is first targeted to the endosomes for Ag loading before traveling to the cell surface (52, 53).

A classic measure of the dependence of MHC class I for peptide is the ability to stabilize the surface molecule by incubation at lower temperatures (30). Therefore, the inability to stabilize empty HLA-F upon cold treatment of cells would argue against peptide binding being involved in stabilization of the complex. However, this latter caveat to peptide binding could be explained by the single exception that we found in the MHC deletion mutant .221. After testing an extensive panel of cells, we found that only LCL .221 showed up-regulation of HLA-F. Therefore, HLA-F expressed in this cell line behaved like other class I in being stabilized despite the fact that HLA-F expressed in parent deletion line .144 was not stabilized. The MHC defect of .144 includes the HLA-A gene and surrounding sequences and is shared by .221, while .221 has additional deletions of the HLA-B and HLA-C genes (29, 31), including some 150 kb of sequences flanking HLA-B (D. E. Geraghty, unpublished observations). Restoration of the HLA-B and -C genes into .221 did not alter the ability of HLA-F to be stabilized by cold treatment, ruling out a direct involvement of these genes in this phenotype. One model for HLA-F expression and regulation that might account for these cell-specific differences is that a negative inhibitory factor is encoded or regulated by a gene(s) found within the regions specifically deleted in .221. This factor is hypothesized to regulate HLA-F protein transport to the cell surface, possibly by binding HLA-F and acting as a retention factor.

It is possible that peptide is bound to one glycosylated HLA-F form and not the other, presumably the Endo H-resistant form binding peptide, and that one form is empty or expressed as a free heavy chain. Precedent for that has been observed in the HLA-B27 homodimer expressed coincidently with HLA-B27 complexed with peptide and β_2-m on some cells (54). However, the distinct forms may instead reflect different pathways in their progression to the cell surface, and both pathways could conceivably provide peptide or other ligand, as is the case with CD1d (52, 53). Given the cell lines expressing surface HLA-F and specific mAbs, these questions may now be more directly addressed.

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